CONTRACTILE PROTEINS OF MARINE INVERTEBRATE SPERMATOZOA [1]

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One of the most striking and significant contributions to our insights into the nature of sperm motility has been the observation that "spermosin," a protein with ATP-ase activity obtained from bull spermatozoa, could enter into a reactive dual protein complex with skeletal muscle actin (Burnasheva, 1958). Employing methods conventionally used in the extraction of myosin and actomyosin, Burnasheva prepared the spermosin which undergoes a reversible combination with rabbit psoas actin. Addition of adenosinetriphosphate decreased the viscosity of the synthetic actospermosin by nearly a factor of two. This dramatic demonstration of the interaction of actin with spermosin not only appears to duplicate the molecular interaction of actin with myosin, but also suggests that the structural specificity of spermosin closely resembles that of the complementary combining sites of myosin for actin. But since, even after prolonged extraction the flagella yield a protein which by itself exhibits no viscosity change in the presence of ATP, Burnasheva concluded that the flagella must lack actin.

However, assuming that, at the molecular level, at least a formal similarity exists between the flagellar movement and muscular contraction, one may suppose that flagella may contain a second protein, analogous to actin, which, interacting with spermosin through the mediation of ATP, participates in the production of the flagellar wave. If this protein were structurally bound in such fashion as to prevent its ready extraction by the same means used to obtain spermosin, an alternative extraction procedure could perhaps release the actin analog from the flagellar organelles.

Treatment with acetone or detergents is frequently useful in freeing such proteins from cell particulates. Watery extracts of acetone powders of flagella isolated from sonicated starfish spermatozoa form a clear gel-like pellet which contains a bound nucleotide (Plowman and Nelson, 1962). Mohri (1964) adapted Gibbons' (1963) use of digitonin to solubilize bonds which apparently hinder the extraction of contractile proteins from sea urchin and fish sperm. The digitonin treatment provided the means for obtaining, almost quantitatively, a highly active ATP-ase in solution in alkaline 0.6 *M* KCl. After dialysis against dilute buffer, further extraction of this protein with cold perchloric acid yielded a substance

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with an adenine nucleotide ultraviolet absorption peak (258 mμ; one of the characteristics of actin is its high content of bound adenine nucleotides). Mohri evidently obtained an actomyosin-like protein from the spermatozoa.

A. G. Szent-Györgyi had previously shown that reagents containing strongly electronegative anions, such as KI, which depolymerize actin, also readily extract the actin from muscle (1951). Since inordinately large volumes of sperm suspensions are needed to prepare the acetone powders (Plowman and Nelson, 1962) and since nonpolar solvents denature myosin, a combination of the Mohri procedure with that of Szent-Györgyi was employed in extraction of flagellar proteins for a study of their viscometric properties.

Materials and Methods

Gonads removed from the arms of mature male starfish, *Asterias forbesi*, were minced in filtered sea water and the spermatozoa poured through four layers of cheesecloth. The spermatozoa were disrupted by sonic oscillation with a Bronwill Ultrasonic Probe at a frequency of 20 kc./sec. for 45–60 seconds in an ice bath. Centrifugation at 1000 *g* twice, for 10 minutes each time, removed most of the heads. The tails could then be sedimented in 10 minutes at 10,000 *g*. The residues were suspended in five volumes of cold 0.6 *M* KI, 0.06 *M* $Na_2S_2O_3$ and 0.1% digitonin (in 2.5 m*M* Tris buffer pH 8.3 and 30 m*M* $MgCl_2$) and extracted for one hour in the refrigerator. After the suspension was centrifuged for 10 minutes at 5000 *g*, the supernatant was decanted and diluted with 15 volumes of ice-cold deionized water and the pH adjusted to 4.65 by addition of 1.0 *M* sodium acetate buffer. The precipitate settled overnight in the refrigerator; excess fluid was removed by aspiration and the precipitate concentrated in the centrifuge at 5000 *g*. Solid $NaHCO_3$ was added to neutralize the precipitate. At this point some of the preparations were dissolved in 0.6 *M* KCl and tested viscometrically for ATP-sensitivity.[2] Other preparations were dispersed in 0.6 *M* KI + 0.06 *M* $Na_2S_2O_3$ + 10^{-4} *M* ATP, using a Teflon homogenizer, and an equal volume of cold deionized water was added. Insoluble material was removed by centrifugation and to the supernatant was added one quarter volume of 95% ethyl alcohol chilled to −20° C. The mixture was then dialyzed against 6.7 m*M* phosphate buffer, pH 7, containing 10 μ*M* ATP, with changes every half hour for four hours; the precipitate was removed and to each 100 ml. of dialysate were added 2 ml. of saturated KCl. This was kept at room temperature for one hour, chilled and then centrifuged in a Beckman-Spinco Model L Ultra-centrifuge #40 rotor for 60 minutes at 35,000 R.P.M. The supernatant was carefully drained off and the clear colorless pellets were dissolved in 2 ml. of 10 μ*M* ATP, 10 μ*M* ascorbic acid. These solutions were tested in Ostwald viscometers in a constant temperature bath at 0° C. Each sample was run through the viscometer at least 5 times, usually at four-minute intervals. Protein concentrations were determined according to the method of Lowry *et al.* (1951).

[2] On the basis of its viscometric response to the addition of ATP, the product at this stage of preparation is referred to as flactospermosin. Subsequent to its treatment with the 95% alcohol and removal of the spermosin, the material is called flactin.

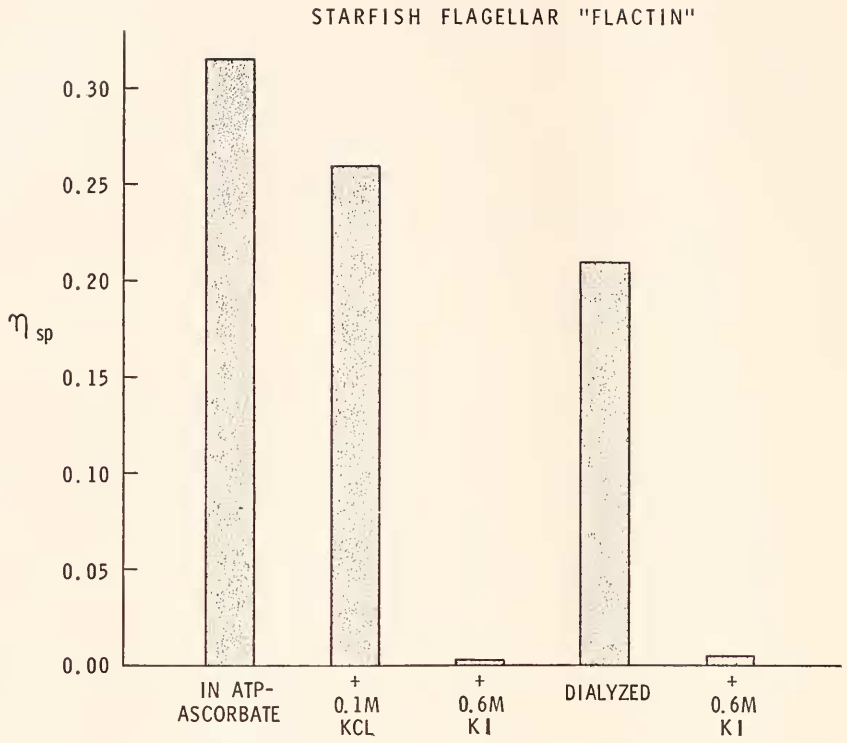

FIGURE 1. Starfish flagellar flactin. Ordinate, specific viscosity of flactin. Two-ml. samples at 0° C. Initial sample prepared from flagellar extract after removal of KI by dialysis against ATP-ascorbate, polymerization in 0.1 *M* KCl and centrifugation at 100,000 *g* to form clear pellets. Pellets dissolved in 10 μM ATP-ascorbate; protein concentration 4 mg./ml. Second bar, addition of 0.2 ml., 1 *M* KCl; no further polymerization. Third bar, addition of 0.2 ml. of 6 *M* KI + 0.6 *M* $Na_2S_2O_3$; depolymerization. Fourth bar, removal of KI by dialysis against ATP-ascorbate; repolymerized. Fifth bar, depolymerization by addition of $\frac{1}{10}$ volume of 6.0 *M* KI + 0.6 *M* $Na_2S_2O_3$.

RESULTS [3]

Viscosity measurements made on the redissolved flagellar "actin" pellets gave outflow times ranging from 125 to 135 seconds, depending on the protein concentration, while additions of KI (final concentration of 0.6 *M*) lowered the time to about 105–110 seconds.

The outflow time of pure solvent (0.6 *M* KI + 0.06 *M* $Na_2S_2O_3$) was 103 seconds and of the ATP-ascorbate (10 μM) was 107 seconds.

In the first experiment (see Fig. 1) the specific viscosities were determined for a sample of the starfish sperm flagellar "actin" which had a protein content of 4–5 mg./ml. Addition of KCl (final concentration = 0.1 *M*) did not cause any ap-

[3] The viscosity terms used in this paper are defined as follows:
Specific viscosity (η_{sp}) = relative viscosity −1, where relative viscosity is viscosity of the protein solution divided by solvent viscosity.
Reduced viscosity (η_{sp}/c) = specific viscosity divided by the protein concentration.
Intrinsic viscosity = the limit of (η_{sp}/c) as c approaches zero.

parent further polymerization; in fact the outflow time decreased slightly. However, 0.2 ml. of 6 *M* KI + 0.6 *M* $Na_2S_2O_3$ added to the 2-ml. sample caused up to a 25% decrease in outflow time. The specific viscosities had dropped from 0.315 to 0.260 (KCl) to 0.04 (KI), pointing to a possible depolymerization and presumably conversion from a fibrous to a globular form.

When the samples retrieved from the viscometers were dialyzed overnight against cold 10 μM ATP in 6.7 m*M* phosphate buffer pH 7, the flagellar "actin" or "flactin" apparently reverted to the more fibrous form. This brought the outflow time back up to about 126 seconds and a specific viscosity of 0.21. Addition of 1/10 volume of 6*M* KI + 0.6 *M* $Na_2S_2O_3$ again caused an apparent depolymerization, dropping the outflow time to 106 seconds and the specific viscosity to 0.03.

In other samples of the flactin with slightly lower protein content, the KI caused a fairly rapid initial depolymerization followed by a slower small additional decrease in viscosity. Here again, dialysis against the ATP-phosphate buffer restored the viscosity almost to the initial value.

A rough estimate of the intrinsic viscosity of the flactin may be calculated by dividing the specific viscosity of the depolymerized flactin by its concentration. The specific viscosity of the sample in Figure 1 after treatment with KI was 0.04 and the concentration was about 0.4 g./100 ml., giving about 0.1 for the intrinsic viscosity. According to Tsao (1953), who was studying skeletal muscle actin, this reflects the condition of a solution containing particles of considerable asymmetry. Several determinations of reduced viscosity of a flactin preparation (ordi-

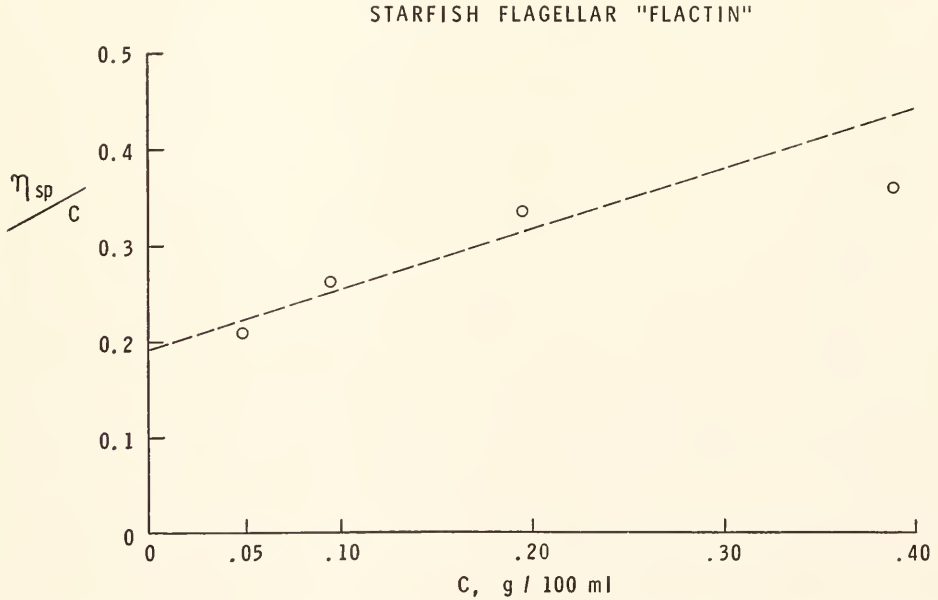

FIGURE 2. Starfish flagellar flactin. Intrinsic viscosity. Ordinate: Reduced viscosity; abscissa: concentration of flactin in g./100 ml. Samples in 10 μM ATP-ascorbate. Extrapolation to zero protein concentration gives value of about 0.2, indicating fair degree of asymmetry of flactin. Viscosity measured at 0° C.

nates: η_{sp}/C) at different concentrations of the sample dissolved in 10 μM ATP-ascorbate solution provide the data for the graph (Fig. 2). Protein concentrations, in g./100 ml., appear on the abscissa. Extrapolation of the curve to infinite dilution of the protein gives an intrinsic viscosity of about 0.2.

Samples dissolved in 0.6 M KCl prior to the step at which spermosin was removed by denaturation in cold alcohol were tested for sensitivity to ATP. This material, referred to as "flactospermosin," had ATP-ase activity and underwent precipitation after 10-fold dilution with 0.1 mM $MgCl_2$ in the absence of ATP and superprecipitation on dilution in the presence of 0.2 mM ATP.

Two aliquots of flactospermosin in 0.6 M KCl had outflow times in the Ostwald viscometers of between three and four minutes. Addition of 1/10 volume of 2 mM ATP-ascorbate solutions caused rapid drops in the outflow time followed by slower

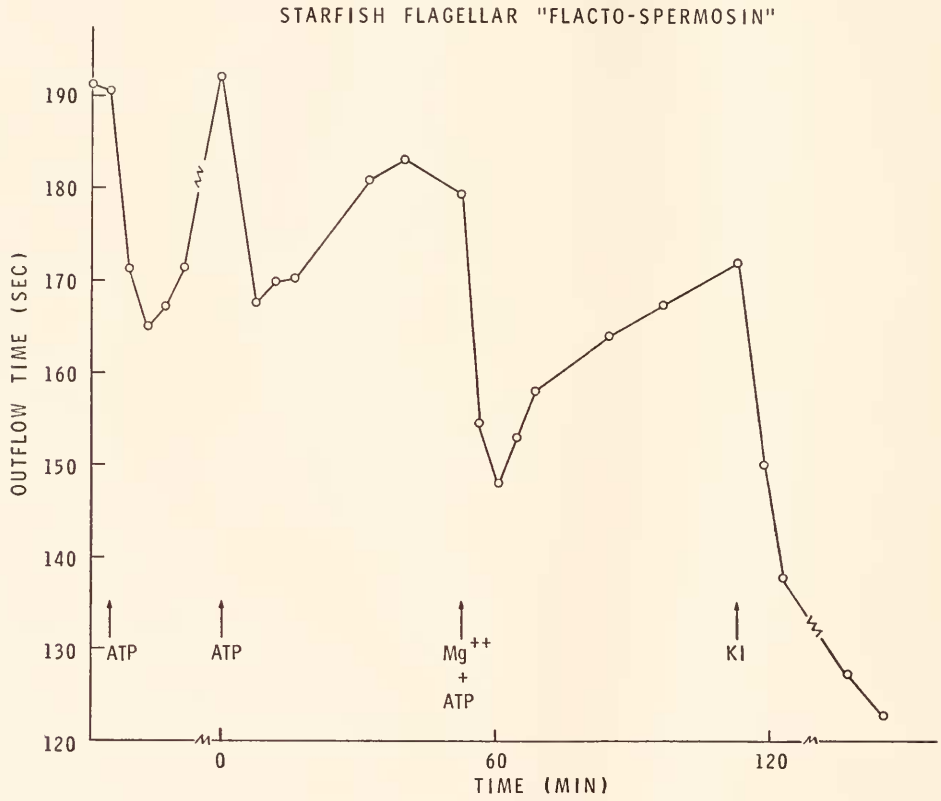

FIGURE 3. Starfish flagellar flactospermosin. Effect of ATP on viscosity of 0.6 M KCl solutions of flactospermosin. Two-ml. samples of flactospermosin; 1/10 volume of 2 mM ATP added; 1/10 volume of 4 mM $MgCl_2$ added at arrow. Ordinate: outflow time in Ostwald viscometer. Abscissa: duration of experiment in minutes to indicate relative time required for "contraction" and "relaxation" showing ATP-sensitivity of flactospermosin. In terms of specific viscosity, deviations from the initial value of 0.83 subsequent to additions of the various reagents and recoveries resulted in the following minima and maxima: 0.57, 0.84, 0.59, 0.74, 0.41, 0.64 and finally 0.17.

recovery to, or nearly to, the initial viscosity. Figure 3 graphically illustrates the course of one of these experiments. The ordinate shows the outflow time in seconds, in the Ostwald viscometer, at 0° C. The abscissa represents the time scale with readings usually at four-minute intervals and also shows the relative amount of time required for the effect of ATP addition and recovery. (The breaks in the abscissa, however, indicate two-hour intervals.) Addition of 0.4 m*M* $MgCl_2$ along with the ATP caused a somewhat greater drop in viscosity, followed by a delayed and less complete reversion to the initial viscosity. This may be due to a slight diminution of the ATP-ase activity of the system caused by the Mg^{++}, since the viscosity drop, which could be attributable to dissociation of the flactin-spermosin complex, depends on the presence of unhydrolyzed ATP, and 1 m*M* $MgCl_2$ inhibits the ATP-ase activity of muscle proteins under these conditions. Finally, 0.6 *M* KI appeared to disrupt the interaction of the dual proteins in this complex, probably through depolymerization of the flactin, which completely prevented the recovery process.

Discussion

While this is intended, not as definitive, but primarily as a progress report, the results appear sufficiently interesting to merit presentation at this juncture.

Burnasheva established that mammalian spermatozoa contain a "contractile" protein, spermosin, which, possessing ATP-ase activity and combining reversibly with muscle actin, physiologically resembles muscle myosin in these respects. Spermatozoa thus may avail themselves of the potential for utilizing interaction between two proteins for producing and propagating a flagellar wave. So far, some of the positive evidence adduced for the existence of an actin-like protein in mammalian sperm has been based on immunocytochemical studies (Nelson, 1962; Nelson and Plowman, 1963). This work, currently being reinvestigated, seems to be substantiated (Young, personal communication). However, recently published reports suggest that fowl actin may not be antigenic in rabbits but that "so-called antiactin antisera are directed against native and classical tropomyosin and perhaps other proteins involved in contraction and relaxation of artificial actomyosin" (Cahn, 1965). On the other hand, Pepe (1966), using chicken breast muscle, prepared what purports to be an anti-actin distinct from anti-tropomyosin, for immunocytochemical studies on chicken myofibrils. In our laboratory, rat actin and rat myosin served as antigens in immunocytochemical analyses of rat epididymal sperm.

The bulk studies on the actin-like component of the contractile system of the sperm have mainly centered on the starfish material because of its availability in greater quantities than either rat or bull sperm, although currently such studies on the mammalian material are in progress.

One of the complicating features in the investigation of actin properties, referred to above, has been the variable degree of contamination with tropomyosin. Special precautions and modifications in the conventional acetone methods for extraction and purification of the actin may reduce this to a minimum (Carsten and Mommaerts, 1963; Baranyi, Baranyi and Guba, 1957). In the present study, because these methods inactivate the ATP-ase, no effort has been made to avert this problem, but while no evidence concerning the possible occurrence of a tropomyosin-

like substance in sperm flagella has come to light, a preliminary diagnostic check by the method of Szent-Györgyi and Kaminer (1963) indicates the strong possibility for the existence in very small amounts of a "tropospermosin" (Kaminer, personal communication). While this in itself is an interesting development and of course opens up new lines for investigation on sperm contractile proteins, nevertheless, it need not detract from the material at hand. In any event, flactin does not hydrolyze ATP, and the experiments illustrated in Figure 3 depend on retention of ATP-ase activity by the flactospermosin.

As previously reported (Plowman and Nelson, 1962), the flactin showed a predisposition to salt out when the pellets were dissolved in media of ionic strengths above 0.05. Presumably aggregation accompanies polymerization as the KCl concentration approaches 0.1 *M*. Attempts to determine sedimentation constants in the analytical ultracentrifuge reflected the tendency of the flactin to aggregate; a fairly sharp peak would form and disappear rapidly before the centrifuge attained a speed of 25,000 R.P.M. This phenomenon will be examined in detail. In ATP-ascorbate (10 μM) or following dialysis against the 6.7 mM phosphate buffer + 10 μM ATP employed to repolymerize the flactin after depolymerization in KI, the solutions were slightly opalescent. During viscometry, the flactin would flow freely and reproducibly, to within fractions of a second, without indication of particle formation.

Intrinsic viscosity, determined following dilution of individual flactin preparations to decreasing protein concentration in ATP-ascorbate, calculated out to 0.2 by extrapolation to zero concentration. This closely approximates that obtained by Tsao (1953) for the monomeric form and is a little less than half his value for a postulated dimeric form, of skeletal muscle actin. Tsao further, allowing for a contribution of hydration to the asymmetry factor (0.39 g. H_2O/g. protein) and assuming a partial specific volume of 0.74, concluded that the actin monomer has an axial ratio of about 12, as a prolate ellipsoid of revolution.

The experimental results so far indicate that sperm flagella contain a second protein (spermosin being the first), associated with configurational changes in molecules attendant upon the formation of the propulsive undulatory wave. This protein, flactin, appears to undergo polymerization readily, to interact reversibly with spermosin, may be asymmetrical in shape, and lacks the ability to split ATP. Other evidence which may support these conclusions lies in the dilution-precipitation behavior of the flactospermosin extracts and the effects of dilution on sea water or isotonic KCl suspensions of isolated sperm tails. In the first instance, dilution-precipitation followed by super-precipitation in the presence of ATP characterizes actomyosin sols. Suspensions of *Mytilus* sperm tails also react in this fashion (Nelson, 1959), but addition of ATP markedly delays the precipitation of diluted starfish sperm tail suspensions, as if the ATP were serving as a relaxing agent. However, to the contrary, ATP expedites the precipitation of the diluted starfish sperm extract, flactospermosin. Speculation on the basis of this apparent discrepant behavior would probably be premature although we have observed some suggestive differences in the motility characteristics of the sperm of *Mytilus, Asterias* and other marine invertebrate species.

While these findings point to a possible formal similarity between sperm tail bending and muscle shortening at the level of protein-protein interaction, ATP

and divalent cation shifts, no attempt should be made to impose morphological identity on the respective fibrous elements concerned. At the fine structure level, one need not invoke a sliding filament mechanism for flagellar action, even though Satir (1964) reasons that sliding filaments must serve as the basis for ciliary action since he could see no fibers shorter than others in electron micrographs of lamellibranch gill cilia. The immunocytochemical studies show that flactin comprises the cortical region of the coarse longitudinal fibers while the spermosin, in intimate contact with the flactin, occupies the core portion of these same fibers in rat sperm (Nelson and Plowman, 1962). Since only about 3% shortening of the fibers will produce the radius of curvature of the sinusoidal flagellar wave, the necessary configurational change could accompany the interaction of flactin and spermosin in the presence of ATP.

Summary

1. Starfish sperm flagella prepared by ultrasonic disruption were extracted with digitonin and 0.6 *M* KI.

2. Flactin, a flagellar protein analogous to muscle actin, was obtained which undergoes viscosity changes similar to depolymerization and polymerization. Like actin, flactin does not hydrolyze ATP.

3. Flactospermosin, however, like actomyosin, does split ATP, and also precipitates when diluted 5–10-fold with deionized water. If ATP is present, superprecipitation occurs. In 0.6 *M* KCl solutions of flactospermosin, ATP causes a rapid drop in viscosity, which reverts to the initial value as the ATP becomes hydrolyzed.

4. The intrinsic viscosity, reduced viscosity extrapolated to infinite protein dilution, of flactin has a value of about 0.2, indicating that this is a fairly asymmetric molecule.

5. While spermatozoa appear to possess the potential for employing a dual protein complex in flagellar wave production, this does not imply that, on a fine structural level, a sliding filament mechanism underlies flagellar movement. Upon interaction of the flactin and the spermosin, ATP hydrolysis could induce the necessary configurational change.

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